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Mouse Lipocalins (MUP, OBP, LCN) Are Co-expressed in Tissues Involved in Chemical Communication

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Chemical communication is mediated by signal production and signal perception and in house mice (*Mus musculus*), both processes involve lipocalin proteins (OBP, MUP, LCN) that transport volatiles and protect them in tissues where they are produced. However, potential roles of lacrimal, nasal, and salivary lipocalins are still not well known. We aimed to determine the expression of the recently described family of odorant binding proteins (*Obp*), along with major urinary proteins (*Mup*) across different tissues in wild mice (*Mus musculus*) to assess the importance of these proteins based on their quantity in particular expression sites. We performed qPCR analysis of selected *Mup*, *Lcn, Obp* genes, and predicted *Obp* members to study their expression in selected tissues. We identified new members of the mouse odorant binding protein gene family in two subspecies, *M. m. musculus* and *M. m. domesticus*. We show that *Mup4* and *Mup5* from the phylogenetically older group-A are co-expressed with *Obp*s in orofacial tissues. We also identified a sexually dimorphic pattern of female-biased *Obp7* and male-biased *Mup4* expression in lacrimal glands. OBPs, MUPs, and LCNs are produced in parallel, which may function to widen the spectrum of bound ligands, potentially including the degradation products of olfactory signals and/or toxic compounds. Moreover, our study demonstrates that several pheromone transporters from the lipocalin family are co-expressed in the nasal and lacrimal tissues of mice with the newly detected OBPs that further expand the already diverse mouse lipocalin family.

Keywords: lipocalin, odorant, chemical communication, *Mus musculus*, olfaction

BACKGROUND

John Maynard Smith and David Harper defined signal as "...any act or structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved" (Maynard Smith and Harper, 2003). The house mouse (*Mus musculus*) uses a system of volatile pheromones (Mucignat-Caretta et al., 2010) and their transporters from the lipocalin protein family that together form a signal (Novotny et al., 1985). Because volatiles degrade in water solutions (Kwak et al., 2013), their life span largely depends on lipocalins that protect them (Hurst et al., 1998; Timm et al., 2001), and transport them in secretory fluids (Flower, 1996) to an outside world. The signals have strong effects on the reproductive success

of the signaler (Thonhauser et al., 2013) due to strong effects on reproductive physiology of the receiver (Whitten et al., 1968; Roberts et al., 2004; Stopka et al., 2007; Janotova and Stopka, 2011) through chemosensory receptors of the main olfactory and vomeronasal organs (Moss et al., 1997; Luo and Katz, 2004).

Since the discovery of the structure and function of olfactory receptors GPCRs—G-protein coupled receptors (Buck and Axel, 1991), research on chemical communication has concentrated on signal reception by nasal and vomeronasal chemosensory neuronal receptors, and on lipocalin transporters of pheromones. Lipocalins generally function to sequester hydrophobic volatiles and transport them in their eight-stranded beta barrel structure (Timm et al., 2001; Sharrow et al., 2002). Volatiles specifically bind to receptors of chemosensory neurons when released (Tirindelli et al., 1998; Novotny, 2003). In mice, the functions of lipocalin transporters are not well understood and most studies focused on the major urinary proteins (MUPs), which are expressed in the liver and transport volatile odor/organic compounds (VOCs) to the urine (Shahan and Derman, 1984; Shahan et al., 1987a,b; Stopková et al., 2007). MUPs have also been reported to be expressed in several tissues other than the liver (Shaw et al., 1983; Shahan et al., 1987a; Cavaggioni et al., 1999; Utsumi et al., 1999; Karn and Laukaitis, 2011), though their functions are not understood.

Mup genes have recently duplicated in rodents, and in house mice they form a cluster of 21 coding genes (and a similar number of pseudogenes), which can be divided into two groups, the group-A (ancestral), containing *Mup3, Mup4, Mup5*, Mup6, *Mup20* (or "Darcin"), and *Mup21* and the group-B, consisting of 15 other *Mup*s sharing almost 99% sequence identity: *Mup1, Mup2, Mup7-Mup19* (Logan et al., 2008; Mudge et al., 2008), reviewed in Janotová and Stopka (2009), Stopková et al. (2009), and Phelan et al. (2014). The level of urinary MUP production is socially regulated in C57BL/6 laboratory mice (Stopka et al., 2007) and wild living *M. m. musculus* (Janotova and Stopka, 2011) and *M. m. domesticus* (Cunningham et al., 2013) mice. Furthermore, male *M. m. musculus* up-regulated urinary MUP production when caged with a female, but down-regulated when caged with a male. Down-regulation of MUPs was more pronounced in males that were defeated in a male–male encounter (Janotova and Stopka, 2011). Furthermore, social experience of parents can regulate MUP expression level in subsequent generations through epigenetic effects (Nelson et al., 2013).

Specific roles limited to a single urinary MUP were attributed only to a major urinary protein MUP20 (or "Darcin"; a group-A MUP) expressed in males, which attracts females and aids spatial learning (Roberts et al., 2010, 2012). Remaining MUPs were supposed to present an individual "barcode" signal due to differences in urinary MUP profiles (Hurst et al., 2001). However, a recent study with sufficient sample sizes shows that MUP profiles of wild male house mice (*M. m. musculus*) are not individually unique. They are not highly stable but instead are dynamic over time with significant changes after puberty and during adulthood (Thoß et al., 2015), thus challenging the "barcode" hypothesis. Moreover, the variation in pheromone affinities of the urinary MUP isoforms provides low support for the proposal that heterogeneity in MUPs plays a role in regulating profiles of available pheromones (Sharrow et al., 2002).

Another group of lipocalins that is thought to be involved in chemical communication, but less understood, is a cluster of the odorant binding protein genes (*Obp*). *Obp* genes have also undergone a series of duplications in mice, and they occur in a cluster of six genes and two pseudogenes on the X chromosome (Stopková et al., 2009, 2014, see **Figure 1A**). Whilst the *Mup* genes are abundant only in house mice and rats (*Rattus norvegicus*) and rarely found in other species of mammals in multiple copies, *Obp* genes occur as a cluster in various mammalian taxa, e.g., porcupines (*Hystrix cristata*) (Felicioli et al., 1993), bank voles (*Myodes glareolus*) (Stopkova et al., 2010), elephants (Lazar et al., 2002), cows (*Bos taurus*) (Bignetti et al., 1985), boar (*Sus scrofa*) (Spinelli et al., 1998; Nagnan-Le Meillour et al., 2014), and potentially also mole rats (*Fukomys anselli*, *F. kafuensis*) (Hagemeyer et al., 2011). One OBP member (i.e., Aphrodisin) has been shown to be major pheromone transporter in vaginal flushes of hamsters (*Cricetus cricetus*). Interestingly, pigs have OBPs and SAL. SAL is the major salivary protein in pigs with affinity to steroids and to 2-isobutyl-3-methoxypyrazine, it is phylogenetically close to MUPs and is expressed by the male submaxillary glands (Marchese et al., 1998). Moreover, three of the six predicted OBP members described (Stopková et al., 2009, 2014) were also corroborated with MS techniques in the tear and saliva proteomes of the laboratory mouse C57BL/6 (Karn and Laukaitis, 2015), though the authors did not further specify detected OBP variants. Therefore, one of the aims of our paper was to detect potential expression sites of mRNAs coding OBPs that were found in the mouse saliva.

Because mice typically begin social interactions by investigating facial and mouth areas (Luo et al., 2003), we may assume that tear and salivary lipocalins secrete chemical signals, whilst nasal and vomeronasal lipocalins activate and/or deactivate chemosensory GPCR receptors. In addition to their function in chemical communication, some lipocalins also have important roles in innate immune responses (Fluckinger et al., 2004; Stopková et al., 2014). We have previously suggested that chemical communication and immunity have been shaped by similar evolutionary forces because the nasal cavity is a place of pathogen recognition via lymphoid tissues and signal perception via chemosensory neurons (Stopková et al., 2009, 2014). Moreover, lipocalins may have as yet another function. The "toxic waste hypothesis" states that various lipocalins are involved in removing toxic waste from the body (Stopková et al., 2009; Kwak et al., 2011) and that some of the compounds might have been constituting a signal under selection (Stopková et al., 2009). The toxic waste disposal role has been experimentally demonstrated in a recent paper (Kwak et al., 2016) where mice loaded with an industrial chemical, 2,4-di-tert-butylphenol (DTBP) used MUPs for a consequent detoxification (Kwak et al., 2016). To conclude, lipocalins are ubiquitous proteins with diverse functions and multiple sites of their expression.

In this study, we investigated potential differences in the expression of selected lipocalins in two sub-species of the house

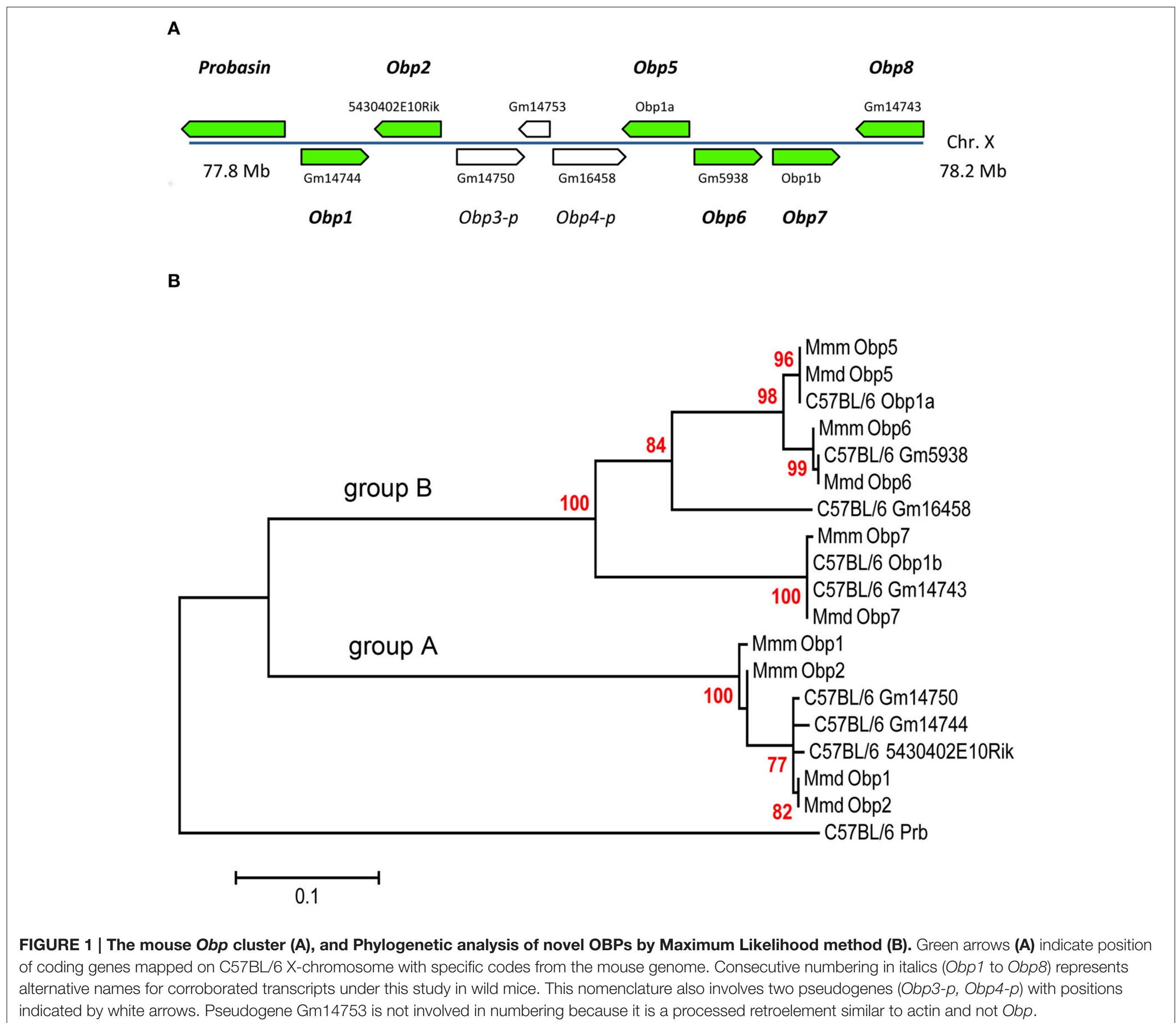


FIGURE 1 | The mouse *Obp* cluster (A), and Phylogenetic analysis of novel OBPs by Maximum Likelihood method (B). Green arrows **(A)** indicate position of coding genes mapped on C57BL/6 X-chromosome with specific codes from the mouse genome. Consecutive numbering in italics (*Obp1* to *Obp8*) represents alternative names for corroborated transcripts under this study in wild mice. This nomenclature also involves two pseudogenes (*Obp3-p, Obp4-p*) with positions indicated by white arrows. Pseudogene Gm14753 is not involved in numbering because it is a processed retroelement similar to actin and not *Obp*.

mouse, *Mus musculus musculus* and *M. m. domesticus*. This could be a starting point to determine how these proteins evolve through speciation (Hiadlovska et al., 2013), and their potential influence on sub-species recognition (Smadja and Ganem, 2002, 2008) and aggression (Dureje et al., 2011). These two sub-species have been previously found to vary quantitatively in the abundance of male VOCs (Mucignat-Caretta et al., 2010) and MUP expression between sexes (Stopková et al., 2007) with differences in the beta-barrel residues under selection (Karn and Laukaitis, 2012). Thus, we have identified new members of the odorant binding protein family and focused on the level and locations of expression of soluble lipocalins. It is our hope that investigating lipocalins in different tissues, and not only in the urine, will provide a better understanding of this fascinating and complex family of carrier proteins.

METHODS

Ethical Standards

All animal procedures were carried out in strict accordance with the law of the Czech Republic paragraph 17 no. 246/1992 and the local ethics committee of the Faculty of Science of Charles University in Prague specifically approved this study in accordance with accreditation no. 27335/2013-17214 valid through 2019. Animals were sacrificed by cervical dislocation.

Samples

The total of 12 individuals (i.e., six males and six females) was studied in this experiment with six individual *M m. domesticus* from Hattingen (51°20′39.84″N, 7°12′06.38″E) and Ruther (51°23′01.0″N, 6°57′48.9″E) and six individual *Mus m. musculus* from Jičín (50°28′18.802″N, 15°22′31.667″E). Individual mice

were kept for 6 weeks following capture in the accredited mouse facility with food and water provided *ad libitum* and on a 12:12-h light cycle with lights off at 1900 h. Samples for 2DE were collected from the oral cavity with 100 µl of 0.9% saline water repeatedly flushing in and out with a pipette. Samples were immediately acetone precipitated and used in further steps.

When salivary samples were collected, all specimens were sacrificed by cervical dislocation and tissue samples were collected from each animal. Tissue samples were obtained from preputial/clitorial glands, liver, lacrimal and Harderian glands, submandibular (salivary) glands, olfactory epithelia, vomeronasal organ, and Nasal-associated lymphoid tissue (NALT). NALT is the paired lymphoid organ (Kiyono and Fukuyama, 2004), and it was isolated from the upper mouse jaw by peeling away the palate where NALT was localized bilaterally on the posterior side.

2DE-Page Analysis

Two dimensional polyacrylamide electrophoresis (2DE) was performed with IEF cell (Bio-rad®) and Protean II electrophoresis system. For the first dimension 12 µg of proteins was applied to Bio-Rad 11 cm strips (pI: 3.9–5.1). Isoelectric focusing was performed after passive rehydration at room temperature and run at 50 V for 9 h, 250 V (rapid) for 15 min, 8000 V (rapid) for 1 h, 8000/30,000 V/h, and finished at 500 V until further step. For the second- dimension separation—strips were equilibrated for 10 min in 45 mM Tris base (pH 7.0) containing 6 M urea, 1.6 SDS, 30% glycerol, and 130 mM dithiothreitol, and then re-equilibrated for 10 min in the same buffer containing 135 mM iodoacetamide in place of dithiothreitol. The strips were then placed on Criterion (dodeca) precast 12–20% gels along with unstained molecular standards in a separate well. Second dimension gels were run at constant current—50 mA for 1 h, 100 mA for 1 h and 150 mA for 1.5 h at 10°C. After electrophoresis, the gels were stained with the Colloidal Coomassie G-250 stain (Bio-rad). All spots in the range 15–30 kDa were excised with a Bio-rad Spot Cutter.

MALDI-MS/MS Analysis

The most abundant protein spots were selected for the analysis and excised from 2-DE gels from 12 individuals. Gel pieces were destained by alternative washing steps using 50 mM ammonium bicarbonate and acetonitrile (i.e., provided in detail in **Data Sheet 1**). After destaining, the proteins in gel pieces were incubated with trypsin (sequencing grade, Promega) at 37°C for 2 h. Digested peptides were extracted from gels using 50% ACN solution with 5% formic acid. MALDI- MS/MS analyses were performed on an Ultraflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Peptide maps were acquired inreflectron positive mode (25 kV acceleration voltage) with 800 laser shots. Peaks within 700–4000 Da mass range and minimum S/N 10 were picked out for MS/MS analysis employing LID-LIFT arrangement with 600 laser shots for each peptide.

CHCA was used as the matrix in combination with AnchorChip target to enhance measurement sensitivity. Sample (1 µl) was mixed with matrix solution on the target in a 2:1 ratio. Known autoproteolytic products of trypsin were used for internal calibration of digested peptides. In the absence of these products, an external calibration procedure was employed, using a mixture of seven peptide standards (Bruker Daltonik) covering the mass range of 1000–3100 Da. The Flex Analysis 3.0 and MS Biotools 3.1 (Bruker Daltonik) software were used for data processing.

Data Processing

MASCOT 2.2 (MatrixScience, London, UK) search engine was used for processing the MS/MS data under standard settings with significance threshold $p < 0.05$. Database searches were done against the NCBI protein database (Release 20101113) without taxonomic restriction. Mass tolerances of peptide precursors and MS/MS fragments were set to 60 ppm and 0.7 Da, respectively. Trypsin specificity with possibility of semitryptic cleavage, oxidation (M), carbamidomethylation (C) and pyro-Glu (Q, N-term) as optional modifications and up to two enzyme miscleavages were set for all searches. Protein identifications based on one or more unique peptides with significant score (under the settings—59 or higher) were accepted. See more details in **Data Sheet 1**.

Real-Time PCR Analysis

Immediately after resection, each tissue sample was placed into Eppendorff tube with a mixture of 1 ml of Trizol (TRIzol Reagent–Invitrogen) and glass pellets, and homogenized using a homogenizer (MM200–Retsch). RNA was isolated using standard Trizol protocol and followed by cDNA synthesis using First strand cDNA synthesis kit (Fermentas). RNA was assessed from the ratio of the optical densities at 260 and 280 nm, and the RNA integrity was assessed with 1% agarose gel containing ethidium bromide. One microgram of total RNA (DNase treated) was used for the synthesis of single-stranded cDNA according to a first-strand cDNA synthesis protocol (Fermentas UAB, Vilnius, Lithuania) with RevertAid™M-MulV Reverse Transcriptase and oligo(dT)18 primer.

Real-time PCR was performed on a Light Cycler 480 (Roche Applied Sciences) using specific dual hydrolysation probe method (Universal Probe–Roche Applied Sciences) with the Probe Master kit (Roche) and protocol according to the manufacturer's instructions. Specific primers and their respective probes were designed by Universal probe library software (Roche) using our newly provided (i.e., *Obp*) and NCBI reference sequences. Intron-spanning assay and multiplex PCR condition with reference gene (Gapdh) were selected. The resulting primers and probes are provided in **Table 1**. Moreover, most group-B *Mup*s are almost 99% similar and it is difficult on the level of transcript to find a probe that would differentiate between different group-B *Mup*s. Thus, most urinary *Mup*s including *Mup2* is included in **Figure 3** within the category MUP-B (detected with universal group-B *Mup* primers).

PCR amplification was performed with the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s where fluorescence was acquired, and elongation at 72°C for 5 s. Each sample was measured in triplicate. The data used for calculation are the means of Cq (i.e., cycle of quantitation

TABLE 1 | Primers and probes used in Real-time qPCR analysis.

Gene	Forward primer	Reverse primer	# UPL probe
Atp5b	ggcacaatgcaggaaagg	tcagcaggcacatagatagcc	77
Lcn11	agaacattgtggacctttctt	ggagaagggtgggtcagc	29
Lcn4	aatgtaggaattcgtttgcag	gagagtatggccccaaaagg	82
Mup-B	gacctatccaatgccaatcg	tggataggaagggatgatgg	47
Mup21	gggaaggaactttaatgtaga	ccacaaaagctctcatgctg	110
Mup4	atggcctgagcctccagt	gctgtatcgatcggaagagag	67
Mup5	gaatgaagaatggcctgagc	caccccatgctgtatggaa	67
Obp1	gcgcaccctttacatagctg	acgctctcaggtctccattc	39
Obp5	ggaccatggaaaactgttgc	cagttctccacctctctctatcttg	146
Obp6	cctgtcctgagtaatgatcttct	ctgattccacaagtcatgaggtt	18
Obp7	tcaagcaaaatggacaatgc	tgccatcttcttgcttataccc	114

in Roche software) values of triplicate samples. The variation in triplicate values never exceeded 0.5 Cq in our samples.

The level of mRNA of the target gene (*Obp* etc.,) in each sample was calculated relative to the reference gene (*Gapdh*) amplified in the same well. A calibration curve was generated for each pair of primers using 10-fold serial dilution of cDNA to assess the value for PCR efficiency (E). In all cases E was not lower than 0.9 (i.e., 90% efficiency of PCR reaction). *E*-values were than used in the formula (The Efficiency sensitive model Pfaffl, 2001) used for the calculation of relative expression (RE), i.e., normalized mRNA abundance:

$$RE = (1 + E_{reference})^{Cp_{reference}} / (1 + E_{target})^{Cp_{target}}$$

Non-template and non-RT reactions were used as controls. For the analysis of expression patterns via hierarchical clustering we used R software. Our hierarchical clustering utilized Euclidian distance metric on log2 transformed data and complete linkage method. The mixed-model approaches, ANOVA, *t*-test, Shapiro-Wilk's normality test, and Fligner-Killeen test for testing the homogeneity of variances, were also computed and plotted in R (Venables and Smith, 2009). Data for pI values were cross-checked from multiple online resources (Ensembl Genome Browser—www.ensembl.org/, NCBI) and with our recently obtained sequences. Isoelectric point was calculated with ExPASy (http://web.expasy.org/compute_pi/), whilst the index of hydropathy (i.e., GRAVY index) was calculated with Gravy calculator (www.gravy-calculator.de/). Sequence data are provided as additional **Data Sheet 2**, data for calculation of pI and H are provided in **Data Sheet 3**.

Sanger Sequencing

Various primer sets were derived from predicted sequences of the genome mouse C57BL/6 (Stopková et al., 2009, 2014) and used to amplify *Obp* transcripts. Mixed samples from studied orofacial tissues were used for transcript identification. Finally we set up a pair of primers per transcript giving one clear band in the expected area. These sequencing primers covered the whole region from start to stop codon: *Obp1*- (F—CTC TGAACTCCTTCGGAAGGA, R—AAAAGAATCAGTACC ATGGTAGGA), *Obp5*—(F—CTGTAGAAAAGAAAGTCT TGTACCA, R—CATTCAAAAAAGGAAGATCATGAGA), *Obp6*—(F—AAGTCTTGTGCCATAATGGCAA, R—TCAAAA AAAGGAATAACAGGTCGTA), *Obp7*—(F - TGAACATCT CCAGAGGAGCAA, R—GGAAGAAGAGTTTATAGATTA GGCAA). The products were double sequenced (downstream, upstream) with 3130 Genetic Analyzer, Applied Biosystems using either forward or reverse *Obp* primers and with 5 to 10 technical replicates per transcript. Sequences were analyzed using the Sequence Scanner (Applied Biosystem) software and compared to predicted and known sequences from public database NCBI using BLAST. Novel sequences were deposited in GeneBank with accession numbers provided in the results section.

Phylogenetic Analysis of *Obp*s by Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−1578.6730) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 284 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

RESULTS

Saliva Contains Lipocalins

To detect the expression of lipocalins on the level of proteins, we used the MALDI- MS/MS analyses for protein identification in mouse saliva. In both subspecies, we have identified several abundant lipocalins (**Figure 2**): OBP5 (Odorant binding protein 1a, gi|1835143), LCN11 (Lipocalin 11, gi|154689678), MUP5 (Major urinary protein 5 precursor, gi|113930708), and highly similar group-B MUPs with the most likely identification provided in **Data Sheet 1**. We have also identified a fragment of a putative pheromone transporter VM (Vomeromodulin precursor, gi|70909314) which is a 70 kDa glycoprotein expressed in the posterior septal and vomeronasal glands but not in the mucus of the main olfactory neuroepithelium (Khew-Goodall et al., 1991). The presence of VM (and potentially also OBP5) in the mouse saliva suggests that nasal and oral cavities are functionally connected because proteins expressed

by nasal/vomeronasal tissues are also found in the oral cavity. We have also identified several proteins below 17 kDa size including PIP [Prolactin Inducible Protein—also highly abundant in saliva of the laboratory mouse (Blanchard et al., 2015)], and several unspecified members of ABP (androgen binding protein) family which were recently described in detail in the tear and saliva proteomes of the genome mouse C57BL/6 (Blanchard et al., 2015; Karn and Laukaitis, 2015).

mRNA Sequencing Corroborated Predicted *Obp*s

In contrast to MUPs, the family of OBPs is rather enigmatic with respect to the expression of their predicted members. In studying the poorly-described OBP1a protein found in mouse saliva, we discovered that it is related to a gene cluster that had been incompletely described (Stopková et al., 2014). Therefore, we have sequenced all *Obp* predicted transcripts in wild mice from pooled oro-facial tissues using primers generated from C57BL/6 genomic data and provided specific product names based on their chromosomal position (for MUPs see Logan et al., 2008). All *Obp* transcripts were mapped on the X chromosome of the laboratory mouse C57BL/6 and have been given consecutive names *Obp1-Obp8* (**Figure 1A**). We have also included two pseudogenes (i.e., *Obp3-p, Obp4-p*; classified by Ensembl as unprocessed pseudogenes without a protein product) in our consecutive numbering of *Obp*s as in other rodent taxa these genes may be intact with ORF (i.e., not truncated) and expressed. Thus, we have provided unique *Obp* sequences for feral *M. m. domesticus* (*Obp1*—KJ605385, *Obp2*—KJ605386, *Obp5*—KJ605387, *Obp6*—KJ605388, and *Obp7*—KJ605389), and *M. m. musculus* (*Obp1*—KJ605390, *Obp2*—KJ605391, *Obp5*—KJ605392, *Obp6*—KJ605393, and *Obp7*—KJ605394) and submitted them to GenBank (NCBI).

Phylogenetic Analysis of Novel *Obp* Sequences

All novel OBPs have a feature typical for the entire *Obp* cluster - a specific disulfide bond (Cys38–Cys42), which represents a strong OBP-diagnostic motif CXXXC (Cys-Xaa-Xaa-Xaa-Cys; reviewed in Stopková et al., 2009, 2014). We used our mRNA (i.e., CDS) sequences along with those from C57BL/6 mice to generate the Maximum Likelihood (MLM) tree (**Figure 1B**). The MLM algorithm with 2000 permutations identified *Prb* (Probasin) as the root (i.e., the outgroup to all OBPs). Thus, remaining *Obp*s form two sub-clusters that we decided to name as the group-A and the group-B *Obp*s. Ancestral group-A *Obp*s include *Obp*1 and *Obp*2 (bootstrap = 100). The later evolved group-B *Obp*s include *Obp5*, *Obp6*, *Obp7*, and *Obp8* (bootstrap = 99). The group-B *Obp* sequences perfectly match those predicted transcripts that we extracted from the laboratory mouse genome (**Figure 1A**). However, newly described *Obp1* and *Obp2* from *M. m. musculus* cluster together (bootstrap = 90) and seem to be divergent from *M. m. domesticus* and C57BL/6 (see **Data Sheet 2** for Multiple sequence alignment).

The strong CXXXC motif present in all OBP proteins (including Probasin) is represented by **C**NDD**C** in OBP1 and OBP2, **C**DEG**C** in OBP7 and OBP8, **C**EKE**C** in OBP5 and OBP6. *Obp3-p* pseudogene (if expressed) would belong to the group-A cluster whilst *Obp4-p* would belong to the group-B, **Figure 1B**. Novel *Obp* (transcript) sequences along with the *Mup* sequences downloaded from NCBI were translated and further used for the calculation of hydropathy and pI properties. We also measured the expression of these newly identified genes in numerous tissues.

Hierarchical Clustering Revealed Differential mRNA Expression Across Tissues

We assessed expression of lipocalins in eight tissues using qRT-PCR. We were primarily interested in the location of expression of the newly described OBPs. So we designed primers for all OBPs and for other lipocalins from the list of identified proteins and the VNO-specific LCN4. In the next step, we used hierarchical clustering in R software as a graphical method to show relationships among expression levels of different genes across tissues. Primarily, we focused on the detection of similarities among expression levels in different tissues and averaged across individuals of the two subspecies to cluster particular tissues on the basis of their similar pattern (**Figures 3A,B**).

Hierarchical clustering separated selected tissues according to their pattern of expression into two groups depicted on (upper) X axis in **Figure 3**. Interestingly, in *M. m. domesticus* - olfactory epithelia (OE), lacrimal gland (LG), and nasal-associated lymphoid tissue (NLT) have clustered together in both subspecies (see **Figures 3A,B**), whilst vomeronasal organ (VNO) and other secretory tissues including liver, Harderian gland, submandibular gland, and preputial gland were located on the other branch in *M. m. domesticus* (**Figure 3A**). In *M. m. musculus* VNO clustered together with OE, LG, and NLT (**Figure 3B**), thus suggesting higher VNO activity in this subspecies. This difference is shown in **Figure 3C** where the *M. m. domesticus* matrix is subtracted from that of *M. m. musculus*. The average matrix in **Figure 3D** is a representative matrix with individuals averaged over the two subspecies.

Sexually Dimorphic *Mup*s in the Liver, and Lacrimal *Mup4* and *Obp7*

Mean value of log2 expression levels from the whole data set and standard deviation was 0.759 ± 1.154. Therefore, we opted for a two-fold ($\sim$2 sd) filtering procedure to obtain data with elevated sexual dimorphisms. To our surprise and probably due to the limited sample size, we have detected only three sexually dimorphic genes: the male-biased group-B *Mup*s (i.e., a group of highly similar genes amplified with the primers provided in **Table 1**) in the liver, and unique male-biased lacrimal group-A *Mup4,* and female-biased lacrimal *Obp7*. Only these three groups were further tested.

To control for pseudoreplication of the expression data (i.e., three measurements taken from each individual with the total of 36 measurements) we used a mixed-effect model approach

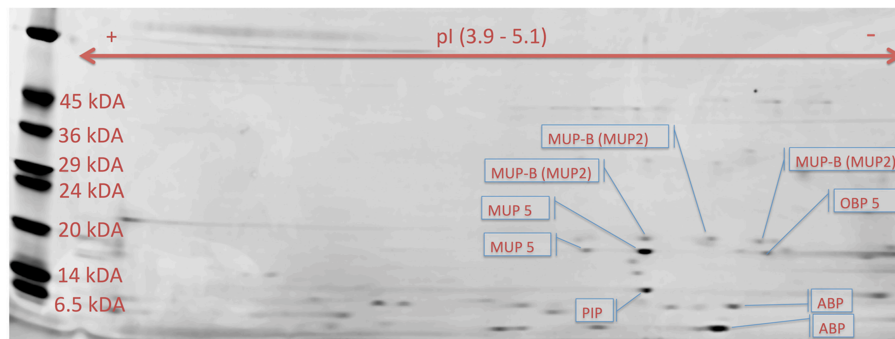
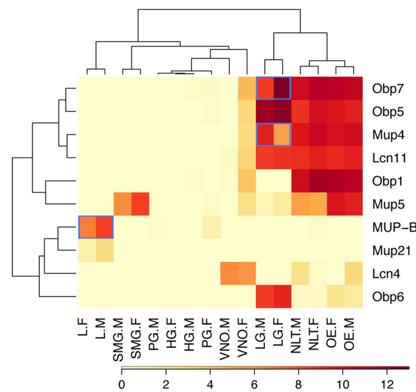
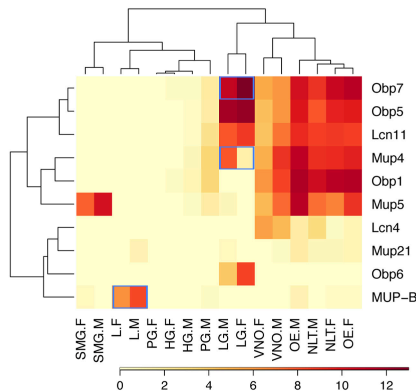


FIGURE 2 | Proteomic search for acidic proteins with hydrophobic beta barrel. Proteomic 2DE analysis on narrow range strips (pI 3.9–5.1) revealed that mouse saliva from a male (*M. m. domesticus*) contains proteins depicted in the figure including OBP5 (MMD11 spot in A1 file), group-A MUP5 (spots: MMD15, MMD17), group-B MUP2 (spots: MMD12-14), unspecified ABPs (spots: MMD18) and prolactin-inducible protein, PIP (spot MMD16). In most gels (i.e., from males and females) we also identified VM (vomeromodulin), LCN11 (lipocalin11). See additional file A1 for a MS report (i.e., section *Mus musculus domesticus*—male).

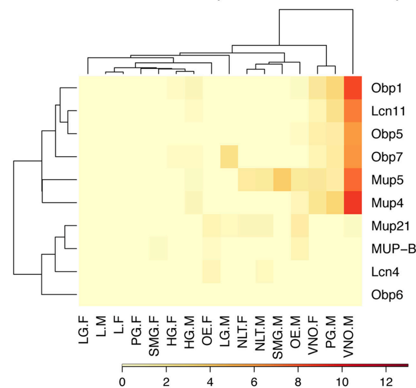
A *Mus musculus domesticus*



B *Mus musculus musculus*



C Distance matrix (*M.m.m.- M.m.d.*)



D Averaged over subspecies

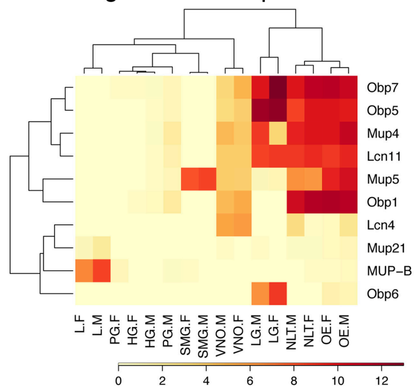


FIGURE 3 | Graphical representation of the qPCR expression pattern. Group-A *Mups* are co-expressed with *Obps* and *Lcns* in sensory tissues and exo-orbital lacrimal glands, whilst the later evolved group-B *Mups* are an outgroup for their specific expression and excretory functions by the liver. Blue-framed are the significantly sexually dimorphic genes (i.e., male-biased group-B *Mups* in the liver, male-biased group-A *Mup4* in lacrimal glands, and female-biased *Obp7* in lacrimal glands). Abbreviations: L, Liver; SMG, submandibular gland; PG, preputial gland; HG, Harderian gland; LG, lacrimal gland; VNO, vomeronasal organ; NLT, NALT / nasal-associated lymphoid tissue; OE, olfactory epithelia; F, females; M, males; Highly expressed genes (~12 fold) are dark red whilst low expression genes are in pale yellow. Heat-maps are provided for each sub-species, **(A)** *M. m. domesticus*, **(B)** *M. m. musculus*, for the distance between them **(C)** and their average **(D)**.

(*nlme* package) assuming normal distribution of the dependent variable, with individuals as random grouping variable (i.e., 12 clusters), and sex, gene, and species as fixed effects. Based on the minimum adequate model (Crawley, 2007) the level of detected sexual dimorphism was highly significant $\Delta df = 9$, L-Ratio $= 102.1$, $p < 0.0001$. *Post-hoc* comparison with Tukey HSD further revealed how each gene contributed to significant sexual dimorphism: *Mus m. musculus*—*Mup*-B ($p = 0.002$), *Mup4* ($p = 0.0002$), *Obp7* ($p = 0.09$ ns); *Mus m. domesticus*—*Mup*-B ($p = 0.009$), *Mup4* ($p = 0.008$), *Obp7* ($p = 0.005$). The data and details of the model are provided in **Data Sheets 2**, **3**. Additionally, we did not detect any significant sex-differences in the expression of lipocalins between *M. m. musculus* and *M. m. domesticus*.

Biochemical Properties of Co-Expressed OBPs and MUPs

Our bioinformatics analysis revealed that OBPs and MUPs have different predicted isoelectric points with MUPs being more acidic than OBPs (2-tailed *t*-test, $p = 0.0009$; **Figure 4**), which differentially affects their solubility at different pH. Data are provided in **Data Sheet 4**.

Instead of looking at particular residues, we searched for a more general parameter that along with the structure directly affects lipocalin-binding properties. As a proxy, we calculated the grand average of hydropathy (i.e., GRAVY values, Xiong et al., 2009) which is defined as the sum of hydropathy values of all amino acids divided by the protein length. The values are negative for all individual members of MUPs and OBPs, however, the spectra of predicted hydrophobicities in OBPs and MUPs are non-overlapping (ANOVA, $F = 54.59$, $p < 0.0001$; Fligner-Killeen test of homogeneity of variances (chi-squared $= 3.474$, $df = 1$, p-value $= 0.06234$).

Furthermore, we have separated the group of MUPs based on previous studies (Logan et al., 2008; Mudge et al., 2008) into the ancestral group-A genes and the later duplicated group-B genes. Statistically significant differences in their mean values are graphically represented in **Figure 4** by non-overlapping confidence intervals. Furthermore, the group-B MUPs evolved hydropathic properties that are intermediate between the group-A MUPs and OBPs.

DISCUSSION

This study attempts to extend the current knowledge on genes for olfactory signals in feral mice by measuring selected mRNA expression across tissues, thus, revealing which other glands and tissues may be involved in chemical communication. We have corroborated that the group-B *Mup* transcripts show sexual dimorphisms in the liver in both subspecies (Stopková et al., 2007) whereby males excrete higher levels of *Mup*s than females. The most interesting result of the current study is a sexually dimorphic pattern of the highly expressed ancestral group-A *Mup4* and the female-biased *Obp7* in lacrimal glands. Tears containing MUP4 and OBP7 are presumably spread onto the fur during a course of facial self-grooming, thus contributing to body odor with hydrophobic ligands that these proteins may transport. Nasal MUP4, however, was suggested to play roles in sequestering pheromones and possibly transporting them to their receptors (Sharrow et al., 2002).

The exocrine roles of lacrimal lipocalins are supported by observations that facial areas elicited strong neuronal activity response in the accessory olfactory bulb (Luo et al., 2003). Similarly *Obp5, Obp6, Obp7* are also highly expressed in lacrimal glands (*Obp5* and *Obp7* originally annotated in inbred C57BL/6 as *Obp1a* and *Obp1b*). Recently, OBP proteins have been identified in tears of the laboratory mouse C57BL/6 (Karn and Laukaitis, 2015). In this study, *Obp* transcripts seem to be co-expressed in combination with other lipocalins (e.g., nasal and lacrimal *Obp5, Obp7, Mup4,* and *Lcn11*). OBP proteins (OBP5 and OBP7) were also predicted to form hetero-dimers (Pes et al., 1992) which may explain why *Obp5* and *Obp7* expression levels cluster together (**Figure 3**). Additionally, lacrimal expression of *Obp7* is female biased and thus, may have female specific roles in chemical communication.

Evaluation of mRNA distribution across tissues also revealed that some lipocalin genes are expressed in just one tissue. Similarly to the group-B *Mup* genes that are mostly expressed in the liver, *Obp6* is expressed only in lacrimal glands, and *Lcn4* is expressed almost exclusively in the vomeronasal organ where LCN4 protein is covering the vomeronasal sensory epithelium to enable primary reception of pheromones (Miyawaki et al., 1994). It is therefore likely, that LCN4 together with Vomeromodulin (Khew-Goodall et al., 1991) and MUP4 (Sharrow et al., 2002) participate in the process of pheromone access and detection by VNO. Following the process of pheromone detection, some of these proteins (LCN3, LCN4, VM, OBPs) are presumably transported to the oral cavity where they are often detected in saliva of C57BL/6 mice (Blanchard et al., 2015; Karn and Laukaitis, 2015) but, as we found in this study, their mRNAs are produced elsewhere (i.e., mainly VNO).

Obp and *Mup* (or *Lcn*) genes are co-expressed in particular tissues probably because their proteins have non-overlapping ligand-binding properties (Cavaggioni et al., 1990) with MUPs having higher and OBPs lower number of hydrophobic residues. This has been originally reported for two OBPs in inbred mice (Cavaggioni et al., 1987; Pes et al., 1992; Pelosi, 1994) and extended for newly detected OBPs in feral mice in this study. Therefore, we also suggest that co-expressed lipocalins may have complementary functions where MUPs may transport more hydrophobic volatiles to and from the vicinity of olfactory receptors whilst OBPs may transport less hydrophobic ligands or may play roles in the deactivation of partially degraded non-specific (i.e., less hydrophobic = hydroxylated or oxidized) volatiles after the signal transduction (Strotmann and Breer, 2011). Our analysis plots in **Figure 4** support such dual functionality. MUPs and OBPs have different pI and therefore may be active under different pH. We have already suggested that this difference in pI may imply that MUPs and OBPs have differential activities during cyclic (de-)

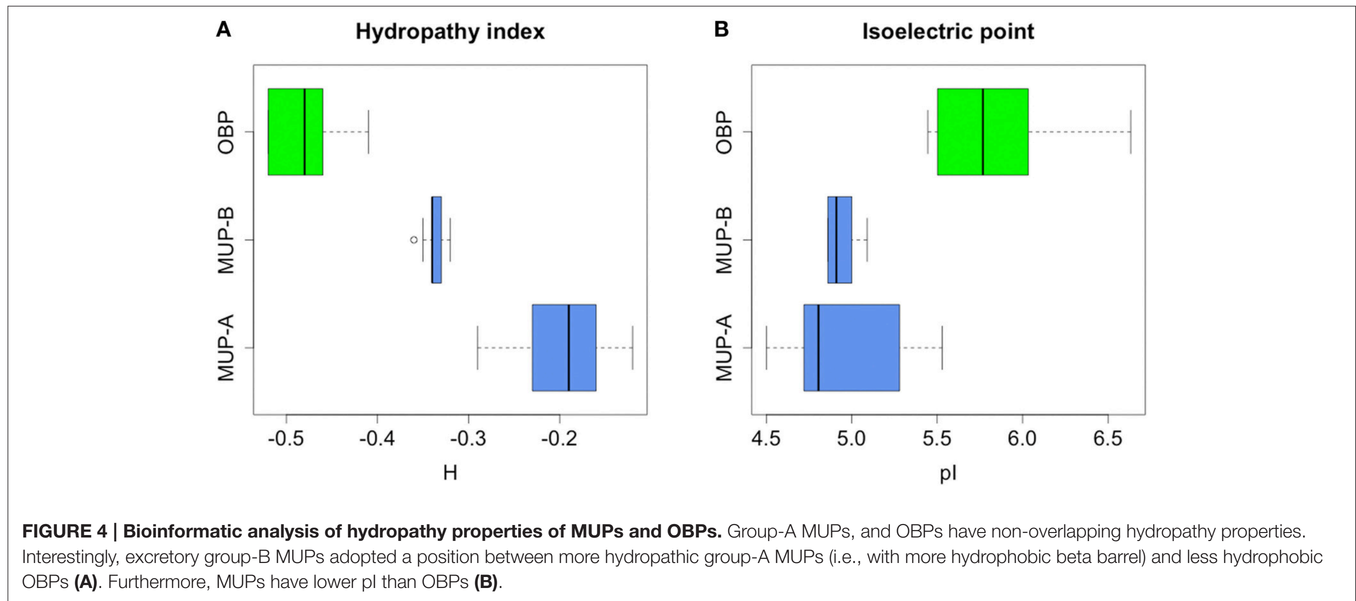


FIGURE 4 | Bioinformatic analysis of hydropathy properties of MUPs and OBPs. Group-A MUPs, and OBPs have non-overlapping hydropathy properties. Interestingly, excretory group-B MUPs adopted a position between more hydropathic group-A MUPs (i.e., with more hydrophobic beta barrel) and less hydrophobic OBPs **(A)**. Furthermore, MUPs have lower pI than OBPs **(B)**.

acidification of nasal mucosa during ventilation (Stopková et al., 2014) similarly as in the study by Cichy et al. (2015) who provided evidence that extracellular pH regulates excitability of vomeronasal sensory neurons. Also, the acidification balance is maintained by Carbonic anhydrase IV (CA IV) which is secreted by salivary, lacrimal, and nasal glands (Kimoto et al., 2004).

The importance of MUPs and OBPs for general olfaction has previously been reported by Sharrow et al. (2002) who analyzed binding properties of nasal *Mup4*, and by Utsumi et al. (1999) who provided evidence that the expression of nasal *Mup* (i.e., most likely *Mup5*) and *Obp* genes is high. Furthermore, many species do not have multiple copies of *Mup* genes and thus MUP products—a major component of chemical signaling and olfaction in mice and rats—but express functional OBPs. This has been shown in many mammals (Singer and Macrides, 1990; Stopkova et al., 2010; Hagemeyer et al., 2011; Nagnan-Le Meillour et al., 2014) and it is our hope that potentially diverse functions—i.e., including the detoxification roles (Stopková et al., 2009; Kwak et al., 2011, 2016)—of these proteins will be further resolved.

CONCLUSIONS

We have studied the expression of known and newly described mRNAs coding for nasal, lacrimal, salivary and urinary lipocalins that are characteristic for their unusually high quantities and the capacity to bind pheromones in their beta barrel. Many of these proteins were individually reported in previous studies by various authors. However, we have provided evidence that some proteins found in saliva are produced by multiple tissues with the normalized expression levels being as high or higher as those described for the urinary group-B *Mup* genes in the liver. For the first time, we have described a novel cluster of odorant binding proteins in feral mice and shown that some of them are differentially expressed in tissues or are sexually dimorphic. Some lipocalins (OBP, MUP, LCN) are co-expressed probably to widen the spectrum of potential ligands that these proteins may sequester and transport. Such expression pattern is almost identical in the two studied subspecies of the house mouse with the exception of VNO, which shows higher lipocalin expression in *M. m. musculus* males. Moreover, further study with sufficient sample sizes could further reveal the level of variation between different individuals and species.

AUTHOR CONTRIBUTIONS

RS performed qPCR analyses and wrote the manuscript. DV and JS were involved in transcript sequencing and qPCR analyses. BK did the bioinformatics analysis of OBP properties. OŠ and ZZ did the MS analyses. TA did most of the statistical analyses. KD was involved in all dissections and sample preparations. PS was involved in designing the experiment, in proteomic analyses and in writing the ms. All authors participated in writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2016.00047

Data Sheet 1 | Protein identification and destaining protocol.

Data Sheet 2 | Multiple sequence alignment of OBPs.

Data Sheet 3 | Original data for statistical analyses.

Data Sheet 4 | Details of the statistical model.

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